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13. ABSTRACT *(Maximum 200 Words)*
The purpose of this award is to identify and characterize an internalization signal in the cytoplasmic tail of prostate specific membrane antigen (PSMA, transmembrane protein abundantly expressed in prostate cancer cells. These studies should aid in understanding the mechanism of antibody uptake and fate of the internalized antibody to improve antibody delivery approaches for immunotherapy for prostate cancer. We have now established and standardized a culture model to study internalization signal of PSMA. In addition we have determined that the cytoplasmic tail of PSMA has an internalization signal. An alanine scan mutagenesis approach in which each of the amino acids in the cytoplasmic tail is mutated to an alanine indicates that a di-leucine motif in the cytoplasmic tail of PSMA may be involved in its internalization. Furthermore, the effect of specific amino acid mutation in targeting PSMA through the endocytic pathway is being tested by immunofluorescence and laser-scanning confocal microscopy. A cell-surface biotinylation assay is being utilized to quantify the internalization of PSMA cytoplasmic tail deletion and point mutants.

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PI - Signature Date

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Introduction

PSMA, a transmembrane glycoprotein of approximately 100kD, is expressed almost exclusively in prostate epithelial cells. PSMA is a type II membrane protein with a short N-terminal cytoplasmic tail and a large C-terminal extracellular domain. Abundance of PSMA in prostate cancer cells and the cell surface localization of this protein make PSMA an ideal candidate for immunotherapy for prostate cancer. Novel PSMA-based prostate cancer therapies, including anti-PSMA monoclonal antibody (mAb)-based therapies are currently being investigated. An internalizing antibody is essential for improvement of immunotherapeutic approaches for prostate cancer. Understanding the mechanism of internalization of these antibodies is a crucial issue in prostate cancer research. Internalized antibody may be targeted to endosomes and recycled back to the cell surface or secreted out of the cell. Alternatively, the internalized antibody may be targeted to lysosomes via endosomes and degraded rapidly within lysosomes. In either case, the effectiveness of the antibody in immunotherapy is dramatically reduced. Prior knowledge of intracellular traffic of the internalized antibody should aid considerably in designing antibody delivery approaches for immunotherapy. Internalization and movement of proteins through endocytic pathway (i.e. targeting to endosomes or lysosomes) are mediated by internalization signal/s present in the cytoplasmic domain of internalized proteins. Anti-PSMA antibody internalization should be mediated by a specific amino acid sequence motif (internalization signal) of PSMA. Identification of the internalization signal of PSMA is crucial to understand the mechanism of antibody uptake and to determine the fate of internalized antibody. The purpose of this research is to identify internalization signal in the cytoplasmic tail that might be involved in targeting PSMA through the endocytic pathway.

Progress Report:

Identification and characterization of an internalization signal of the Prostate Specific Membrane Antigen

We have made tremendous progress in this project designed to understand the internalization signal of PSMA. In the last 12 months of the funding period our goal was to develop a cell culture model to characterize the internalization signal of PSMA and to test whether an internalization signal is present in the cytoplasmic tail of PSMA. In this report we show that we have now determined that an internalization signal is present in the cytoplasmic tail of PSMA using a cell culture model.

Development of the culture model: As indicated in my proposal our plan was to express a green fluorescent protein (GFP) tagged PSMA in LNCaP cells. To do this cDNA encoding full length PSMA was subcloned into EGFP vector (Clontech) and transfected into LNCaP cells by Lipofectamine method. Neomycin resistant cells expressing GFP were selected. During selection, these cells clearly showed fluorescence on the cell membrane as well as in intracellular vesicles as expected for PSMA localization in LNCaP cells. However, the fluorescence gradually diminished in culture and was not detectable for further studies by epifluorescence microscopy. Thus, we were unable to stably express PSMA-GFP fusion protein in LNCaP cells. However, when GFP was expressed alone it was clearly expressed in these cells. We further tested whether this problem is specific to PSMA, by tagging the β-subunit of sodium pump, a type II membrane protein like PSMA. β-subunit-GFP chimera behaved similarly in LNCaP cells indicating an inherent problem to express membrane protein-GFP chimera in LNCaP cells. Due to this problem we resorted to alternate methods to study the internalization signal of PSMA.

We selected COS (African green monkey kidney cells) that are extensively utilized for internalization studies of various proteins. The advantage of this cell line is that it can be transiently transfected with high transfection efficiency so that one can monitor internalization of proteins in transiently transfected cells. COS cells were transfected with full length PSMA cDNA in pCDNA3 vector. 48hrs after transfection, cells were incubated with monoclonal antibody against PSMA (mAb J591) for one hr. Cells were then fixed and permeabilized and stained with a fluorescent secondary antibody. This experiment clearly showed that mAb J591 was internalized in PSMA expressing cells. Double immunofluorescence localization using lysosomal and endosomal markers and laser scanning confocal microscopic visualization techniques were utilized to study the endocytic mechanisms of PSMA in COS cells. These results clearly revealed that PSMA is localized primarily in the endosomes with in 20 min of uptake and in 2 hrs is targeted to the lysosomes as we reported earlier in LNCaP cells (Liu et al., 1998, Cancer Res.58: 4055-4060). These results demonstrated that COS cells could be utilized as a model to characterize the internalization signal of PSMA.

Cell surface biotinylation assay to monitor internalization in COS cells: We then developed a cell surface biotinylation assay to monitor internalization of PSMA in transiently transfected cells. COS cells transiently transfected with full length PSMA was biotinylated using

a cleavable biotin at 4°C. Cells were then transferred to 37 °C for PSMA internalization to take place. After 2hrs of incubation at 37°C, the remaining surface biotin was cleaved by a reducing agent. Biotin cleavage of cells maintained at 4°C served as control. As we reported earlier in LNCaP cells (Liu et al., 1998, Cancer Res.58: 4055-4060) we found that in COS cells ~60% of the total surface PSMA was internalized. Thus we have now established and standardized two assays to study the internalization of PSMA in transiently transfected COS cells: (1) *A morphological immunofluorescence and confocal microscopy assay and 2. A quantitative cell surface biotinylation assay.*

Role of the cytoplasmic tail in the internalization of PSMA: To determine whether the cytoplasmic tail of PSMA contains the internalization signal we deleted the cytoplasmic tail of PSMA by PCR and expressed the cytoplasmic tail deletion mutant in COS cells by transient transfection. Immunofluorescence and confocal microscopy clearly revealed that the cytoplasmic tail deletion mutant of PSMA is not internalized indicating the presence of an internalization signal in the cytoplasmic tail. To further confirm the internalization signal in the cytoplasmic tail we are now in the process of generating a Tac-PSMA cytoplasmic domain (Tac-PSMACD) chimera. Tac is interleukin-2 receptor α-chain, a non-internalized protein. Internalization of this chimera should further substantiate the presence of an internalization signal in the cytoplasmic tail of PSMA.

Identifying the internalization signal in the cytoplasmic tail of PSMA: To identify the internalization signal in the cytoplasmic tail of PSMA we have generated a series PSMA mutants by alanine scan mutagenesis. In alanine scan mutagenesis approach each amino acid in the cytoplasmic tail of PSMA is mutated by PCR and the resulting mutant is subcloned into pCDNA3 vector as shown below. Mutations were confirmed by DNA sequencing.

1. WT	**M W N L L H E T N S A V A T A R R PR**
2. ALA 2	- **A** -
3. ALA 3	- - **A** - - - - - - - - - - - - - - - - - - -
4. ALA 4	- - - **A** - - - - - - - - - - - - - - - - - -
5. ALA 5	- - - - **A** - - - - - - - - - - - - - - - - -
6. ALA 6	- - - - - **A** - - - - - - - - - - - - - - - -
7. ALA 7	- - - - - - **A** - - - - - - - - - - - - - - -
8. ALA 8	- - - - - - - **A** - - - - - - - - - - - - - -
9. ALA 9	- - - - - - - - **A** - - - - - - - - - - - - -
10. ALA 10	- - - - - - - - - **A** - - - - - - - - - - - -
11. ALA 14	- - - - - - - - - - - - - - - **A** - - - - - -
12. ΔCD	**M A R R P R**

Fig.1. Cytoplasmic tail mutants of PSMA used in this study. WT= wild type full-length cytoplasmic domain. Ala 2 to Ala 14 indicates the position of the amino acid mutated to alaline. DCD= Cytoplasmic tail deletion mutant of PSMA. Amino acids ARRPR are not deleted since they are probably required for the type II topology of PSMA.

The cytoplasmic tail of PSMA contains 3 phosphorylation acceptor sites (amino acids 8, 10 and 14). These amino acids were also mutated to study whether phosphorylation of the cytoplasmic tail is involved in the internalization of PSMA. All these mutants were transiently transfected into COS cells and the their internalization was monitored by J591 uptake, immunofluorescence and confocal microscopy as described above. These results revealed that mutation of the leucine (amino acid #5) of the cytoplasmic tail dramatically decreased the internalization of PSMA. In addition mutation of the amino acid leucine (amino acid #4) also decreased the internalization levels but not as much as alanine 5 mutant. These results indicate that amino acids # 4 and 5 (leucine, leucine) a putative di-leucine motif in the cytoplasmic tail of PSMA may function as the internalization signal of PSMA. Currently we are generating double mutants in which both leucine residues (amino acids 4 and 5) will be mutated to alanine to confirm that a di-leucine motif in the cytoplasmic tail of PSMA indeed mediates its internalization.

We are also characterizing the role of cytoskeletal elements such as actin and microtubules that are involved in the internalization of PSMA in prostate cancer cells and in normal epithelial cells. We anticipate that in the next six months we will have sufficient data to publish these results. We predict that these results should aid in the development of novel strategies that may aid application of anti-PSMA antibodies for the immunotherapy of prostate cancer.

Key Research Accomplishments

- ✓ **Cell culture model for studying the internalization signal of PSMA**
- ✓ **Morphological immunofluorescence and confocal microscopic internalization assay in transiently transfected cells**
- ✓ **Biochemical cell surface biotinylation assay for quantification of the internalization transiently transfected cells**
- ✓ **Determination of the presence of an internalization signal in the cytoplasmic tail of PSMA**
- ✓ **Generation of PSMA cytoplasmic tail mutants by Alanine scan mutagenesis**

Peer
10/29/2001

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